REVIEW

It's all in your head: new insights into craniofacial development and deformation

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Introduction

'Our ignorance of the laws of variation is profound. Not in one case out of a hundred can we pretend to assign any reason why this or that part has varied. But whenever we have the means of instituting a comparison, the same laws appear to have acted in producing the lesser differences between varieties of the same species, and the greater differences between species of the same genus.' (Darwin, 1859).

Charles Darwin did not gild the truth when he candidly pointed out just how imperfect is our knowledge of how morphological diversity is generated. Over the intervening decades we have scrutinized the process of embryonic development and, in doing so, gained a deeper appreciation for the tissue interactions that lie at the basis of morphogenesis. More recently, some of the molecules involved in mediating these tissue interactions have been identified and, in a few rare cases, we even have gleaned insights into how species-specific development is controlled. These instances, however, are few and far between and much remains to be accomplished.

In this review we will present some of the most recent advances that lie at the heart of understanding the mechanisms controlling normal craniofacial development, the consequences of when these normal pathways are disrupted and how this information sheds light on the basis for evolutionary diversity among the species. We choose to focus on morphogenesis of the craniofacial complex for two reasons. First, faces show tremendous phenotypic variation, first evident during the later stages of fetal development. Yet despite these differences in the facial appearance of embryos, they all look remarkably similar during earlier stages of embryogenesis. This suggests that whatever factors influence craniofacial diversity primarily act during a discrete period of embryonic development; and knowing when diversity first arises is an important first step towards understanding how diversity is generated.

The second reason we use craniofacial development as a model is that variations in the craniofacial complex are tightly associated with adaptive radiations into ecological niches. If the relationship between craniofacial morphology and speciation is causal (and not merely correlative) then perhaps we can understand how modifying the spatial and temporal patterns of gene expression create diversity within a species.

One final justification for using craniofacial morphology as a model system is the prodigious amount of documentation that has accrued on head and neck anomalies. As the noted embryologist William Harvey so eloquently wrote, 'Nature is nowhere more accustomed openly to display her secret mysteries than in cases where she shows traces of her working apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature by careful investigation of cases of rarer forms of disease' (Harvey, 1657). Thus, thoughtful inspection of the malformed face may offer valuable clues into the mechanisms governing normal craniofacial morphogenesis.

Components of the craniofacial complex

In its postnatal form the vertebrate head has an intricate and highly varied morphology, but during early stages of embryonic development it exhibits a much more simple geometry (Fig. 1). There are seven prominences that comprise the vertebrate face: the midline

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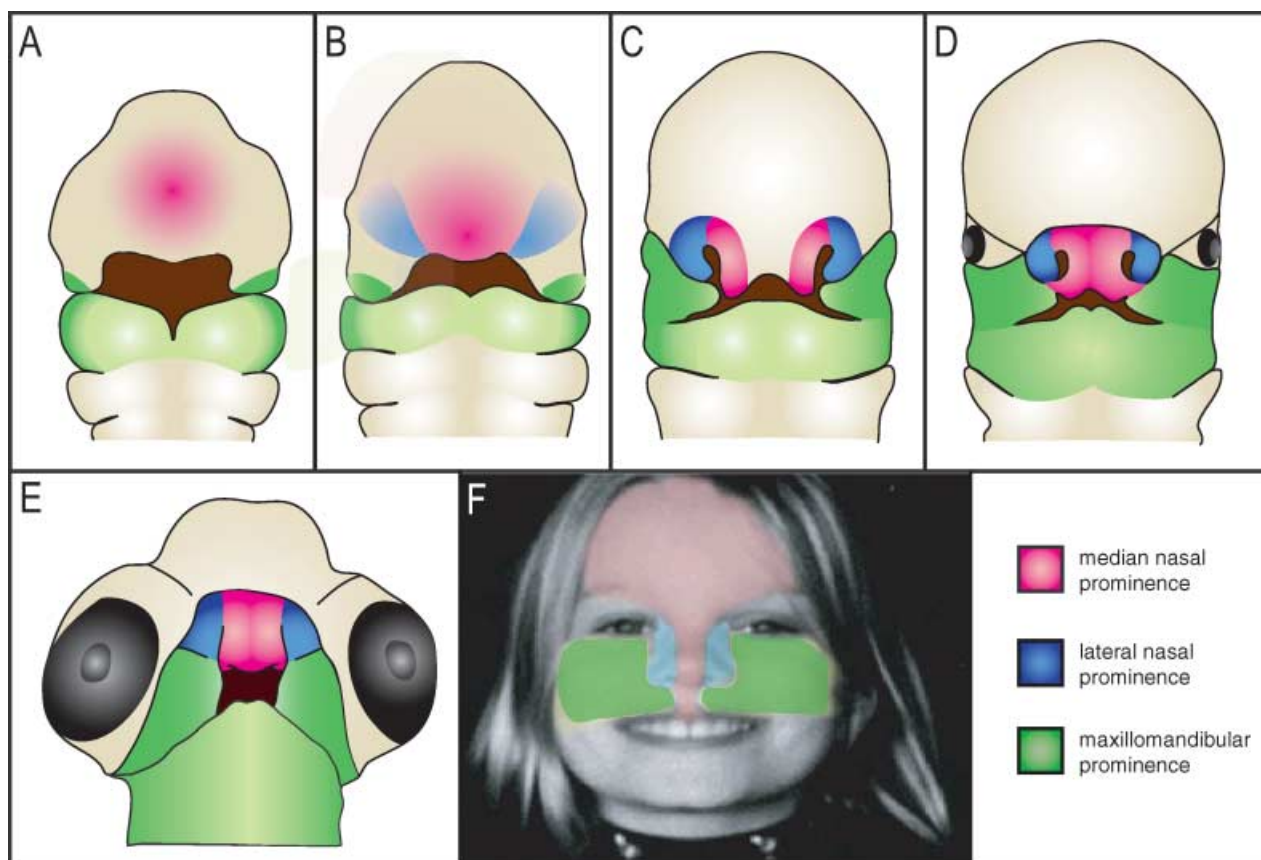
Fig. 1 Development of the craniofacial primordia. (A–D) Representations of frontal views of mouse embryos showing the prominences that give rise to the main structures of the face. The frontonasal (or median nasal) prominence (pink) gives rise to the forehead (A), the middle of the nose (B), the philtrum of the upper lip (C) and the primary palate (D), whereas the lateral nasal prominence (blue) forms the sides of the nose (B,D). The maxillomandibular prominences (green) give rise to the lower jaw (specifically from the mandibular prominences), to the sides of the middle and lower face, to the lateral borders of the lips, and to the secondary palate (from the maxillary prominences). (E) Frontal view of a chick embryo, also showing which prominences give rise to different facial structures. (F) Frontal view of a human child, with different facial structures colour-coded to indicate the prominences from which each structure developed.

frontonasal prominence, and three paired structures, the lateral nasal, maxillary and mandibular prominences (Fig. 1D–F). These maxillary and mandibular prominences are derived from the first pharyngeal (branchial) arch, whereas the frontonasal prominence is derived from a midline primordium that forms on top of the forebrain. The frontonasal prominence contributes to the forehead, middle of the nose, philtrum of the upper lip and primary palate (Fig. 1). The lateral nasal prominence forms the sides (ala) of the nose; the maxillary prominences contribute to the sides of the face and lips, and the secondary palate; and the mandibular prominences produce the lower jaw (Fig. 1). Disruptions in the rate, the timing or the extent of outgrowth of any of these prominences will adversely affect the fusion process. Consequently, one can appre-

ciate the wide variety of facial clefts that occur (Tessier, 1976) and also why facial clefting is the most frequently occurring head and neck birth defect (Perrotin et al. 2001).

Craniofacial patterning and the neural crest

Each of the pharyngeal arches, and the frontonasal prominence, is composed of mesenchyme surrounded by epithelia. In the case of the frontonasal prominence, the mesenchyme is derived from the cranial neural crest, and the epithelia encasing the prominence include the neuroectoderm of the forebrain, and facial (surface) ectoderm (Fig. 2E,F). In the maxillary and mandibular prominences, the encasing epithelia are derived from facial ectoderm and pharyngeal endo-
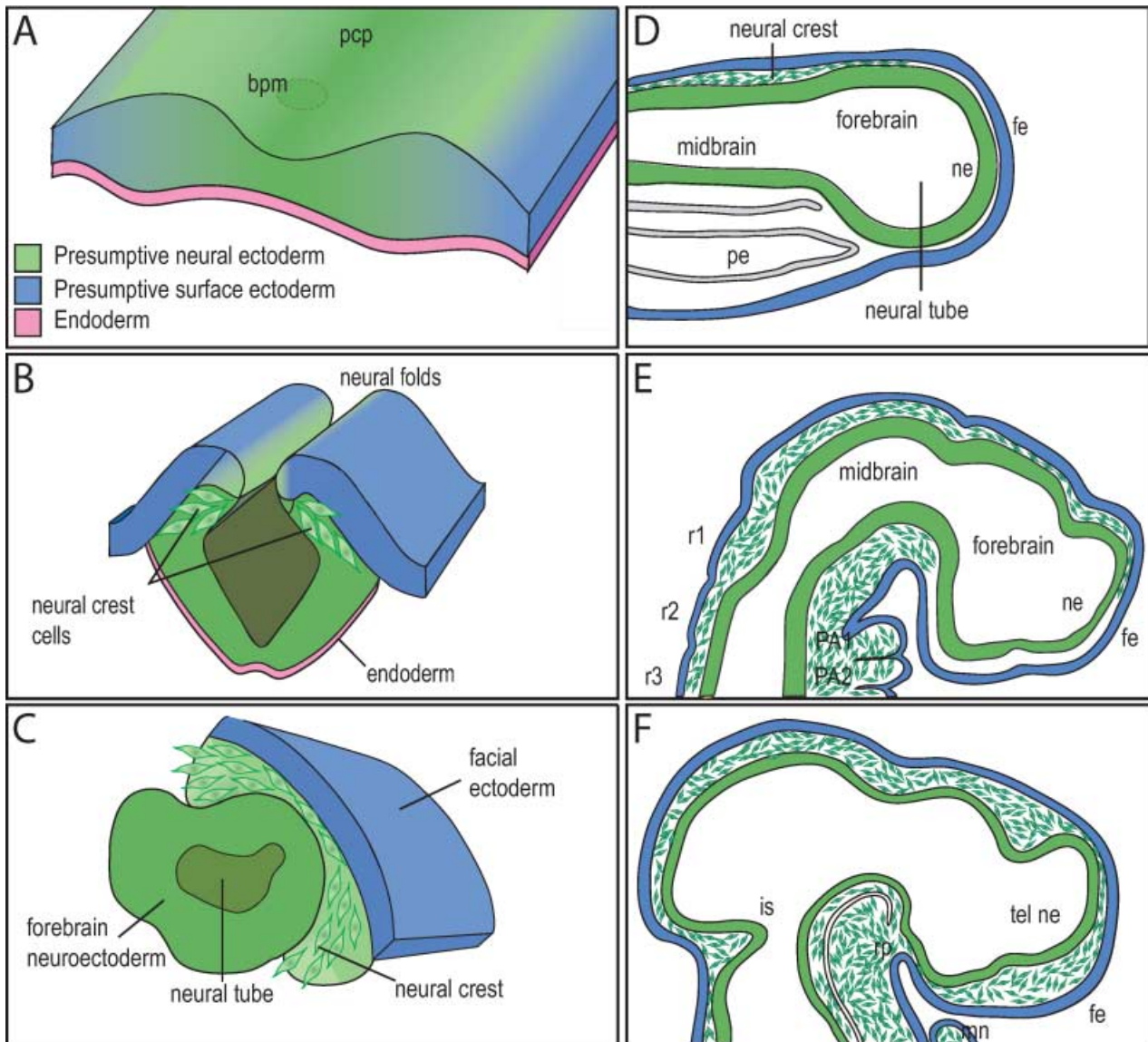
Fig. 2 Neural crest induction and migration in the developing embryo. (A) The neural plate consists of a unified layer of ectoderm, beneath which lies the endoderm. The neural folds arise as the ectoderm begins to fold upwards. Interactions between signalling molecules cause the medial portion of ectoderm to begin to assume a neural character (green) while lateral portions of ectoderm begin to take on a non-neural character (blue). The prechordal plate mesendoderm (pcp) and the buccopharyngeal membrane (bpm) are indicated. (B) As the neural folds begin to fuse, the neural tube takes shape, giving rise to distinct tissue layers of neuroectoderm (green) and surface ectoderm (blue). Neural crest cells start to delaminate from the border region between the neuroectoderm and surface ectoderm. (C) Once the neural tube has closed, neural crest cells lie interposed between the facial (surface) ectoderm (fe) and the neuroectoderm (ne). (D–F) As the central nervous system begins to form from the neural tube, the neural crest starts to migrate anteriorly from rhombomeres (r1–r3) into different areas of the face, and into the pharyngeal arches. Abbreviations: C, caudal; is, isthmus; mes, mesencephalon; mn, mandible; PA, pharyngeal arch; pe, pharyngeal endoderm; rp, Rathke's pouch; R, rostral; tel ne, telencephalic neuroectoderm.

derm, whereas the mesenchyme is derived from both cranial neural crest and mesoderm.

The cranial neural crest cells populating each arch arise from distinct anterioposterior positions along the neural axis (Kontges & Lumsden, 1996), and once resident in the arches, proliferate in a highly regimented fashion. In some cases, the instructions for this prolifer-

ative activity are inherent within the neural crest cells themselves; in other cases, the surrounding epithelia provide the directives. For example, neural crest cells in which *Homeobox transcription factor* (*Hox*) genes such as *Hoxa2* are expressed are constrained, to a certain degree, in their ability to respond to local cues from overlying or underlying epithelia (reviewed in Le

Douarin et al. 2004). Cells devoid of *Hox* gene expression appear much more responsive to signals emanating from the epithelial environment, even into very late stages of morphogenesis (reviewed in Helms et al. 2005). Some of these epithelial cues have been identified and in the next sections we will review these molecular pathways and what is known about their roles in craniofacial morphogenesis.

Not all neural crest cells play by the same rules

The presence or absence of a particular *Hox* code in a neural crest cell determines the extent to which neural crest cells are able to respond to cues from the surrounding milieu (reviewed in Trainor & Krumlauf, 2000; Le Douarin et al. 2004). Other neural crest cells, such as those destined for the frontonasal prominence, do not express *Hox* genes, and therefore our laboratory set out to determine the extent to which these cells were restricted in their developmental potential; or, phrased another way, the extent to which these cells exhibited plasticity and thus were able to respond to local cues from the facial ectoderm (Schneider & Helms, 2003).

To test whether neural crest cells inherently possess directions for facial patterning, we began by exchanging neural crest cells between quail and duck embryos (Schneider & Helms, 2003) (Fig. 3). We specifically targeted neural crest cells destined for the upper beak for two reasons. First, the transplanted neural crest cells were *Hox* negative and therefore we would be assessing the plasticity vs. pre-patterned status of neural crest cells separate from the function of *Hoxa2*. Second, the short, narrow quail beak and the long, broad duck bill meant that we could use differences in the morphology of duck and quail faces (Fig. 3A) as a readout of whether neural crest cells contained patterning information.

After neural crest cells were exchanged between duck and quail embryos the chimeras were allowed to develop to the stage where morphology of their beaks was evident (Schneider & Helms, 2003). Duck embryos that received grafts of quail frontonasal neural crest cells exhibited short quail-like beaks ('qucks') (Fig. 3B) whereas quail embryos that had received transplants of duck neural crest cells had duck-like bills ('duails') (Schneider & Helms, 2003) (Fig. 3C). These findings suggested that neural crest cells direct their own morphogenesis according to instructions inherent in

the donor population (Schneider & Helms, 2003). To understand the molecular basis for these morphological transformations, we performed the same types of grafting experiments and then used molecular and cellular analyses to determine how the grafted cells acted in their new environment and, in turn, how the environment influenced the behaviour of the transplanted neural crest cells. We found that transplanted neural crest cells maintained the temporal gene expression patterns of their original environment despite being transplanted into a new site. In addition, the transplanted neural crest cells altered the temporal pattern of gene expression to reflect the donor, and not the host, environment (Schneider & Helms, 2003).

The same conclusion was reached by Abigail Tucker and Andrew Lumsden, who independently performed similar types of interspecies transplants (Tucker & Lumsden, 2004). They, too, found that the capacity to form species-specific skeletal elements in the head was an inherent property of the neural crest, and concluded that this characteristic is articulated is response to signals from epithelia (Tucker & Lumsden, 2004). In fact, other new experiments directly show that most neural crest cells acquire at least some patterning information from nearby epithelia (reviewed in Helms et al. 2005).

The extent to which facial features were transformed was directly proportional to the number of transplanted neural crest cells that made their way into the chimeric tissue. In other words, the transformation was a 'population-dependent' effect, quite analogous to previous transplantation studies (reviewed in Helms et al. 2005). So it seems that when the contingency is large enough, neural crest cells follow molecular cues that are generated and maintained by the assemblage itself, and disregard signals emanating from the local environment. Just what these population-dependent cues are, and how many cells are required to maintain them, is completely unknown.

The nature of prespecification

Hox genes are probable mediators of the population-based behaviour exhibited by neural crest cells (Capecchi, 1997; Barrow & Capecchi, 1999). In the craniofacial region, *Hox* genes are expressed by neural crest before and after migration to the arches (Hunt et al. 1991b,c; Wilkinson, 1993; Favier & Dolle, 1997; Couly et al. 1998; Rijli et al. 1998; Trainor & Krumlauf, 2000, 2001;
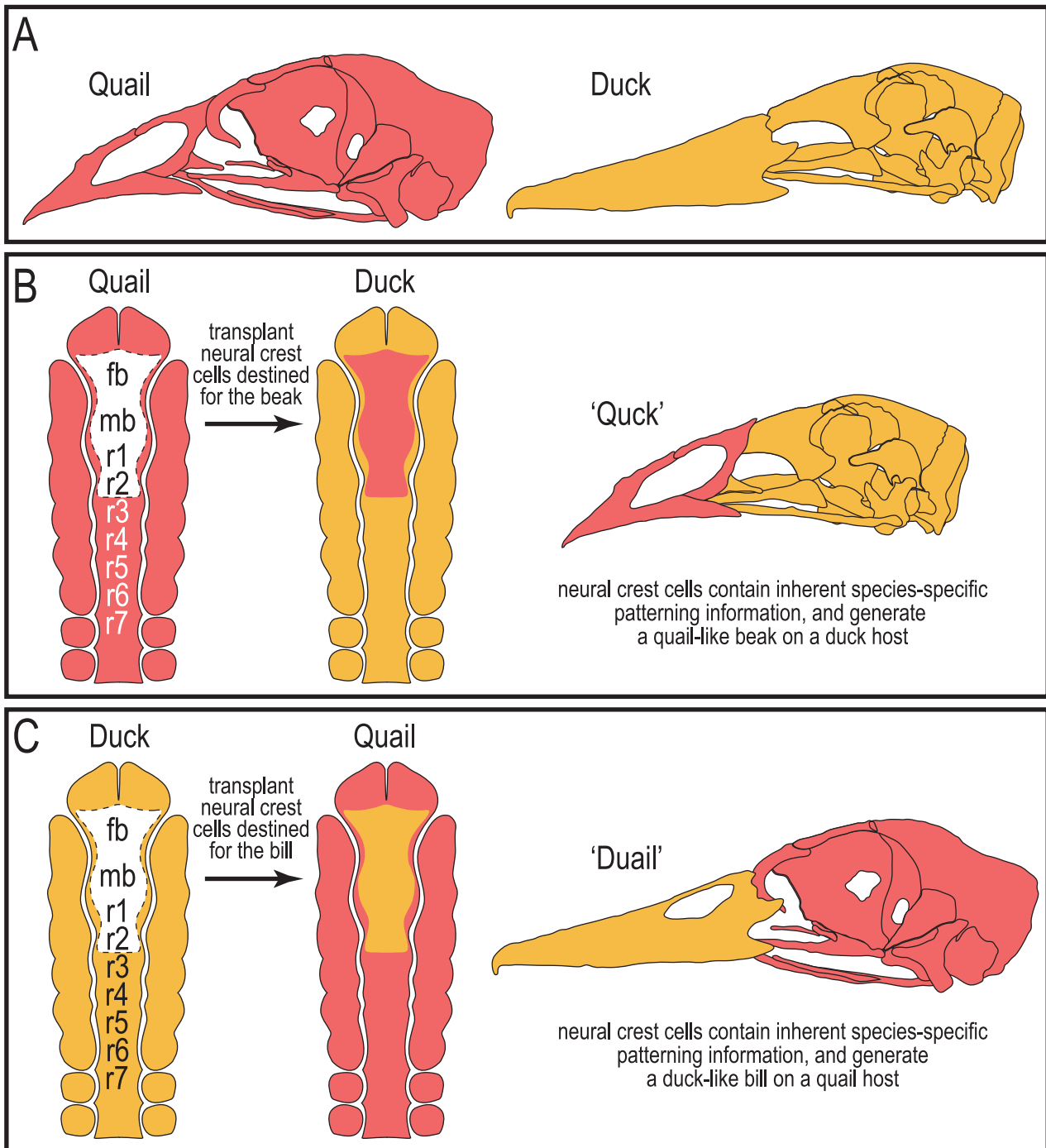
Fig. 3 Transplantation experiments provide evidence that the neural crest inherently contains species-specific patterning information. (A) Quail and duck embryos exhibit distinct anatomical features. For example, quails exhibit a shorter, narrower beak compared with the longer, broader duck bill. (B) When quail neural crest cells from forebrain (fb), midbrain (mb) and rhombomeres 1 and 2 (r1, r2) are transplanted into a duck host, a quail-like beak develops in lieu of a duck's bill. (C) When duck neural crest cells are transplanted into a quail host, the quail develops a duck-like bill. (Figure reproduced courtesy of *Nature*.)

Trainor, 2003). These domains are preserved even after neural crest cells take up residence in the branchial arches (Hunt et al. 1991a,b).

After migration, *Hoxa2*-positive neural crest cells occupy the second and more posterior arch mesen-chyme, which gives rise to the hyoid bone and other structures (Creuzet et al. 2002) (Fig. 4A). *Hoxa2* is not expressed in crest cells of the first arch in most vertebrates (Creuzet et al. 2002) (Fig. 4A). This distinct expression boundary of *Hoxa2* has led to speculation
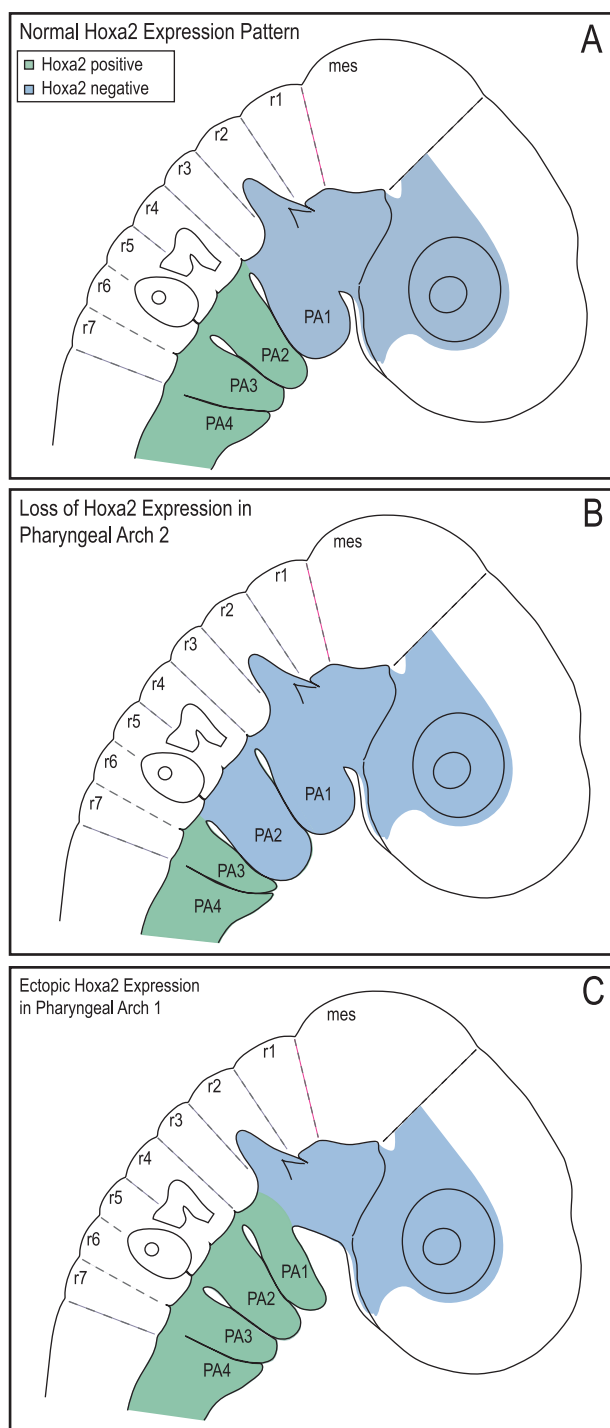
Fig. 4 Manipulation of *Hox* expression. (A) In jawed animals, *Hoxa2* is expressed up to pharyngeal arch 2 (PA2). (B) Loss of *Hoxa2* expression in pharyngeal arch 1 (PA1) gives neural crest cells in PA1 increased plasticity, and they undergo transformation to a first arch fate. (C) When *Hoxa2* is ectopically expressed in PA1, neural crest cells in this arch take on second arch fates, giving rise to a duplication of the hyoid arch.

that neural crest cells may be prespecified by virtue of the expression of this *Hox* gene.

Gain- and loss-of-function studies bolster this hypothesis. For example, loss of *Hoxa2* from the second arch allowed the second arch to take on first arch character, eventually resulting in duplication of maxillary and mandibular structures (Gendron-Maguire et al. 1993; Rijli et al. 1993) (Fig. 4B). By contrast, over-expression of *Hoxa2* in all tissues of the first arch caused the first arch to take on a second arch character (Grammatopoulos et al. 2000; Pasqualetti et al. 2000) (Fig. 4C). Additionally, if *Hoxa2* is transfected into the rostral domain of the cephalic neural crest, these cells lose their ability to differentiate into skeletal structures (Creuzet et al. 2002). Transplantation and ablation experiments lend further confirmation that *Hoxa2* expression confers upon neural crest cells an anterioposterior identity (Couly et al. 1998; Ruhin et al. 2003).

Craniofacial epithelia as sources of instructive signals

Given that the *Hox* transplantation and ablation experiments demonstrated that some neural crest cells are capable of responding to local cues from the surrounding environment, the question then becomes which tissues in the environment are 'talking' to the neural crest. Once the neural crest delaminates from the surface ectoderm during neurulation (Fig. 2B), it lies sandwiched between several epithelia: the surface ectoderm, the neuroectoderm and the pharyngeal endoderm (Fig. 2C–F). Its close contact with these epithelia during development (Fig. 2D–F) allows these tissues to provide instructive signals that help to pattern the neural crest. Recent studies have shed light on the role of the surface ectoderm in initiating outgrowth of the frontonasal prominence, and studies on pharyngeal ectoderm have revealed the importance of endoderm in patterning of the pharyngeal arches. The neuroectoderm also has a significant influence on patterning the neural crest into the craniofacial skeleton, as blocking molecular signals from the neuroectoderm leads to craniofacial syndromes such as holoprosencephaly (HPE).

Surface ectoderm as a source of craniofacial patterning information

Are neural crest cell fates dictated by patterning information inherent in this population of cells, or do neural
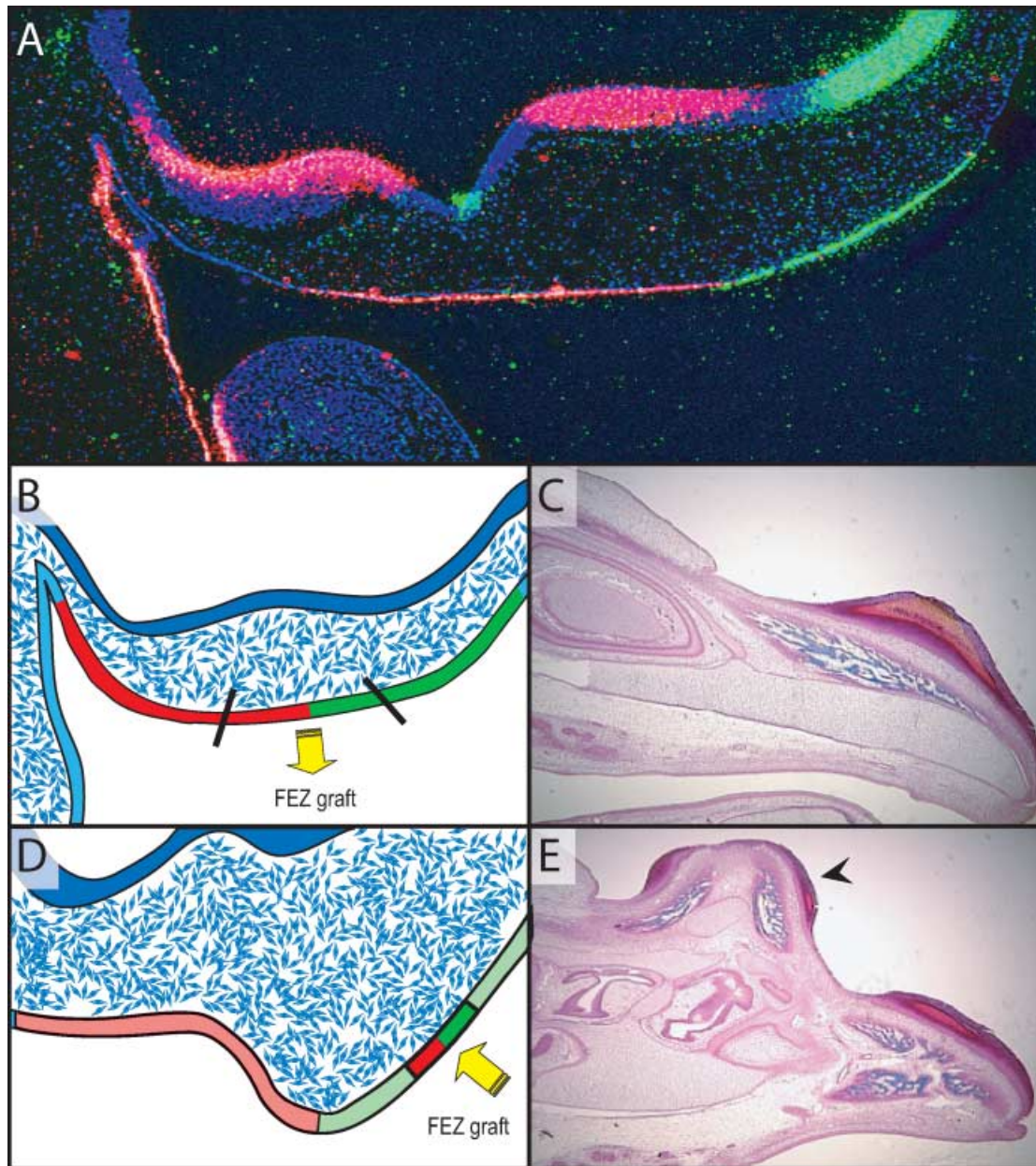
Fig. 5 Transplantation experiments reveal that the facial ectodermal zone (FEZ) is crucial for patterning of the frontonasal primordia. (A) *In situ* hybridization on a sagittal section of a stage-20 chick embryo; red corresponds to an *Shh*-expressing domain and green corresponds to an *Fgf8*-expressing domain. (B,D) Representations of similar lateral sections of donor stage-20 (B) and host stage-25 (D) chick embryos, respectively. (C,E) Trichrome-stained sections of stage-36 control and transplanted embryos, respectively. When the FEZ is transplanted (yellow arrows) from a stage-20 chick donor (B) to a stage-25 chick host (D), an ectopic beak forms by stage 36 (E, black arrowhead). Donor FEZ tissue is indicated by darker red and green colouring, while host FEZ is indicated by lighter red and green colouring.

crest cells respond to signals from their local environment? Studies from our laboratory also suggest that in the frontonasal prominence, the surface ectoderm provides instructive signals that influence neural crest cell fate, even late in development. We identified a region of facial ectoderm that was delineated by the gene expression boundaries of *Fibroblast Growth Factor 8* (*Fgf8*) and *Sonic Hedgehog* (*Shh*) (Fig. 5). The junction of the dorsal, *Fgf8*-positive domain and the ventral, *Shh*-positive domain coincided with the tip of the upper beak, such that the dorsal (top) surface was derived from *Fgf8*-expressing epithelium and the ventral (inside) surface arose from *Shh*-expressing epithelium (Hu et al. 2003). We termed this junction

between *Fgf8* and *Shh* the frontonasal ectodermal zone (FEZ).

Not only does the FEZ demarcate the initial site of frontonasal outgrowth, it also is responsible for setting up the dorsoventral axis of the upper beak. We showed this by ectopically transplanting the FEZ to a more dorsal position of the frontonasal prominence (Fig. 5B–E). In this new site, the FEZ induced a molecular cascade that re-programmed the fate of neural crest cells at the transplant site (Hu et al. 2003). The net result was duplications of upper beak structures (Hu et al. 2003) (Fig. 5D–E). We found that transplantation of the FEZ to the mandible also induced the duplication of lower beak structures, indicating that the neural crest cells in both locations remained highly responsive to signals emanating from the facial ectoderm (Hu et al. 2003).

The dorsoventral polarity of the upper beak is also controlled by the FEZ. When the FEZ graft was inverted, the polarity of the ectopic beak structures was also inverted (Hu et al. 2003). Clearly, some neural crest cell fates can be modulated by external cues from surrounding tissues. When the FEZ graft was transplanted to the second (*Hoxa2*-expressing) arch, however, the transplant failed to induce a molecular cascade as we had observed earlier, nor was it able to re-pattern the neural crest cells to duplicate any skeletal structures (Hu et al. 2003). Seemingly, these *Hoxa2*-positive cells disregarded the patterning signals being sent by the transplanted FEZ (Hu et al. 2003). This location-dependent response suggested that the plasticity of a neural crest cell, and its ability to respond to environmental signals, is context dependent (Hu et al. 2003). By extension, these data suggest that removing the influence of *Hox* genes conferred greater plasticity upon neural crest cells.

Pharyngeal endoderm as a source of craniofacial patterning information

Some of the first experimental evidence demonstrating that the pharyngeal endoderm is a source of patterning information came from a study that was originally designed to test the role of neural crest cells on patterning of the pharyngeal skeleton. In this study, the neural tube was ablated prior to neural crest migration (Veitch et al. 1999). Despite the absence of neural crest cells post-ablation, the pharyngeal arches were properly organized (Veitch et al. 1999). These data indicated that neither the formation nor the patterning of the pharyngeal arches was absolutely dependent upon

neural crest cells. The most likely candidate tissue that controlled patterning in this region of the craniofacial complex was the pharyngeal endoderm.

From an evolutionary point of view, pharyngeal 'perforations', the predecessors of the pharyngeal clefts, preceded the development of the neural crest as a cell population (Gans & Northcutt, 1983; Northcutt & Gans, 1983). This type of correlation has been interpreted as indicating that the pharyngeal endoderm was probably the initial source of patterning information. Studies from zebrafish and quail/chick chimeras now offer direct experimental evidence that the pharyngeal endoderm can influence that pattern of the lower face. In zebrafish, the *van gogh* (*vgo*) mutant shows an absence of pharyngeal segmentation and a failure of the surrounding mesoderm to pattern correctly (Piotrowski & Nusslein-Volhard, 2000). Although hindbrain segmentation proceeds normally, the *vgo* pharyngeal clefts do not form. Consequently, neural crest cells exiting from the rhombencephalon fuse in the ventral surface because of the lack of pharyngeal pouches; the end result is a lack of skeletal elements in the pharyngeal region (Piotrowski & Nusslein-Volhard, 2000). This phenotype suggests that the segmentation and differentiation of crest cells to form the pharyneal skeleton is primarily determined by endodermal signalling.

Using the quail/chick chimeric system, Couly, Le Douarin and their colleagues showed that if regions of pharyngeal endoderm were removed then corresponding regions of the facial skeleton were affected (Couly et al. 2002). For example, removing one strip of pharyngeal endoderm resulted in the reduction or absence of the nasal capsule and upper beak; when another strip of tissue was removed then Meckel's cartilage was affected, and when a third strip of tissue was removed then the articular, quadrate and proximal portions of Meckel's cartilage were perturbed (Couly et al. 2002). Transplantation of quail endodermal strips of tissue to an ectopic location resulted in supernumerary lower jaws that were malpositioned immediately above the host jaw, and as we had found for facial ectoderm, the rostrocaudal inversion of these grafts resulted in the ectopic lower jaw developing in an inverted position (Couly et al. 2002). Taken together, these data demonstrate that patterning and orientation of the pharyngeal arch skeleton is dependent upon the endoderm. In turn, the pharyngeal endoderm is able to instruct the *Hox*-expressing neural crest as to the size, morphology and orientation of the pharyngeal skeletal elements.

At least some of the patterning information in the pharyngeal endoderm is mediated via Fgf signaling. In the zebrafish *acerebellar* (*ace*) mutant the loss of Fgf8 results in deformed pharyngeal pouches and the reduction of the hyoid cartilage (Reifers et al. 1998; Draper et al. 2001; Roehl & Nusslein-Volhard, 2001). More recently, Chuck Kimmel and colleagues showed that disrupting *Fg8* signalling results in a complete failure of pouch formation (Crump et al. 2004). Although the pharyngeal endoderm was present in their *Fgf* mutants, the lateral migration of the endoderm was disorganized and consequently the hyoid and branchial cartilages were truncated, similar to the *ace* phenotype (Crump et al. 2004). In mice, *Fgf8* compound heterozygous (*Fgf8*neo/–) mutant embryos exhibit hypoplasia of the first and second pharyngeal arches and their associated clefts (Abu-Issa et al. 2002), and although neural crest cells migrate appropriately into the arches, once they arrive they undergo premature programmed cell death (Abu-Issa et al. 2002).

Fgfs are not the only molecular signal in the pharyngeal endoderm; endothelin-1 (Edn1) is an intercellular signalling molecule that is expressed in the mesoderm of the pharyngeal arches, as well as in the epithelia of the arches. Both mammalian and teleost data indicate that Edn1 is involved in dorsovental patterning of the arches (Schilling et al. 1996; Miller et al. 2000; Remuzzi et al. 2002; Ozeki et al. 2004), perhaps by establishing a morphogen gradient. Downstream targets of Edn1, such as the bHLH transcription factor *Hand2* and the homeobox transcription factor *Bapx1*, are also involved in dorsoventral patterning in the anterior pharyngeal arches (Miller et al. 2003). Specifically, *Hand2* plays a role in specifying the ventral pharyngeal cartilages of the lower jaw and *Bapx1* in specifying the jaw joint (Tucker et al. 2004b).

Sonic Hedgehog and craniofacial patterning

Over the past decade, studies have begun to delve into the precise nature of the environmental cues that influence the neural crest. Many of these studies have implicated *Shh* as a critical factor in regulating craniofacial morphogenesis. Indeed, *Shh* seems almost omnipresent, with expression domains in several epithelia presenting themselves at various stages of development. The dynamic expression of *Shh* in the craniofacial tissues is one indicator of the multiple roles this growth factor plays in modulating normal, as well as abnormal, craniofacial development. Its role in all of these tissues has been examined in mammals (humans and mice), birds, and most recently, zebrafish. Despite the species differences in facial forms, the results have been remarkably consistent.

### *Shh* expression is dynamic during embryonic development

Shh is expressed in the facial ectoderm, the neuroectoderm and the pharyngeal endoderm at various stages during development. In birds, *Shh* is sequentially induced in the brain and subsequently in the face (Fig. 6). At stage 15 (Hamburger & Hamilton, 1951), *Shh* expression in the brain is initially restricted to the ventral diencephalon (Cordero et al. 2004) (Fig. 6B). At stage 17, a new domain of *Shh* is induced in the ventral telencephalon, which is separated from the diencephalic domain by an *Shh*-negative optic recess (Cordero et al. 2004) (Fig. 6C). Subsequently, around stage 20, *Shh* expression is induced in the facial ectoderm (Cordero et al. 2004) (Fig. 6D). These same expression patterns for *Shh* are appreciated in zebrafish as well (Wada et al. 2005).

Removal of avian *Shh* expression reveals its integral role in craniofacial development

Some of the first hints that *Shh* is capable of directing craniofacial development came from ablation studies in avian beaks. Akin to the jaws of fish, upper beaks are derived from the frontonasal prominence and the maxillary and mandibular prominences. When we removed frontonasal ectoderm that normally expressed *Shh* we saw an arrest of maxillary prominence outgrowth, whereas ablation of frontonasal ectoderm that did not express *Shh* had no discernible effect on facial morphogenesis (Hu & Helms, 1999). The importance of ectodermal *Shh* in the development of the midface was confirmed by removing *Shh* itself from the frontonasal process, via a function-blocking blocking antibody (Hu & Helms, 1999). Although skeletal elements formed, their dorsoventral patterning and fusion were disrupted, resulting in facial clefting and midfacial hypoplasia (Hu & Helms, 1999; Rallu et al. 2002; Jeong et al. 2004).

Perturbations in *Shh* signalling in mice and humans result in a wide range of holoprosencephalic defects

Mice with null mutations in *Shh* bear even more drastic defects (Chiang et al. 1996). Targeted gene disruption
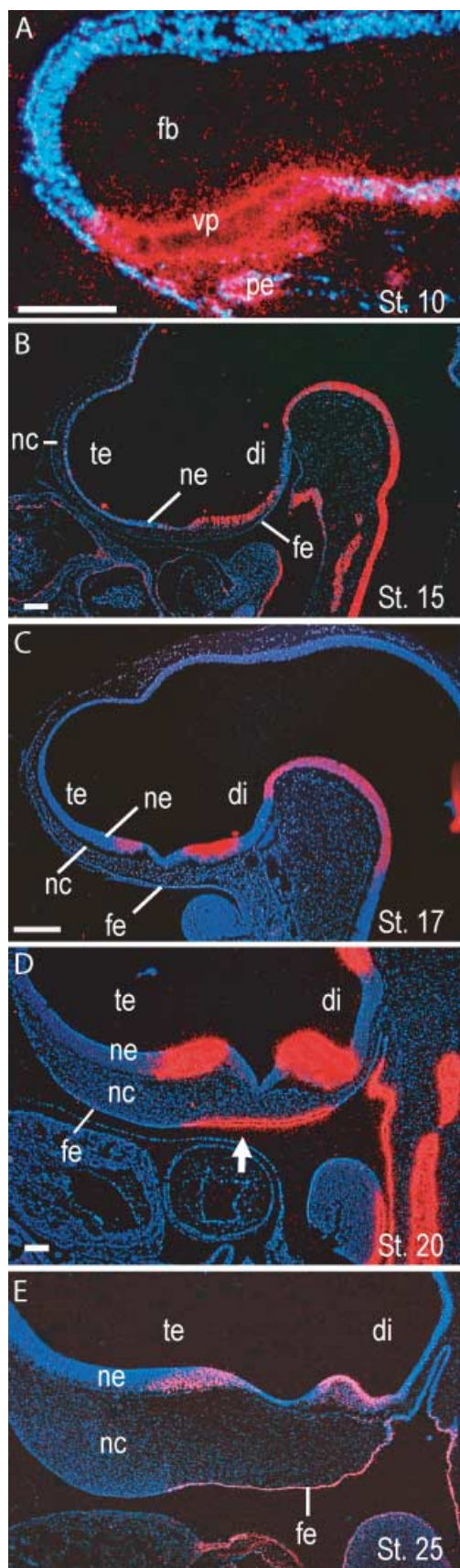
of *Shh* in mice has revealed that it plays a number of critical roles in brain, axial skeleton, limb and craniofacial development (Chiang et al. 1996). Null mutations in *Shh* in murine models lead to significant defects in the establishment and maintenance of midline structures such as the notochord and floorplate at early stages (Chiang et al. 1996). Although most *Shh*-null embryos die early, those that survive to later embryonic stages manifest malformations of the brain, vertebral column and limbs, as well as cyclopia, overall growth retardation and malformations involving the first pharyngeal arch derivatives (Chiang et al. 1996).

Humans with mutations in *SHH* also may exhibit cyclopia, but they exhibit a range of other defects as well. In clinical practice, HPE is traditionally classified into alobar, semilobar and lobar presentations based upon the degree of separation into the individual cerebral hemispheres (Muenke & Cohen, 2000). Alobar HPE is characterized by incomplete separation of the cerebral hemispheres; semilobar HPE is characterized by fairly good separation of the posterior hemispheres but poor separation of the anterior hemispheres; and lobar HPE is characterized by fairly good separation of both posterior and anterior hemispheres (Plawner et al. 2002).

In addition to forebrain malformations, a spectrum of craniofacial phenotypes are seen in human HPE. The most severe facial malformations consist of cyclopia with a proboscis (Situ et al. 2002). Patients in the middle of the spectrum exhibit lesser degrees of facial dysmorphologies consisting of microopthalmia (small eyes), coloboma (incomplete closure of various eye structures, e.g. iris), hypotelorism (decreased interorbital distance), midface hypoplasia (underdevelopmemt

Fig. 6 Sequence of *Shh* expression in a developing chick embryo. (A–F) *In situ* hybridization performed on sagittal sections of chick embryos, where red (pseudocoloured using Photoshop) represents *Shh* expression as obtained by *in-situ* hybridization with S35. (A) At stage 10 *Shh* is expressed in the forebrain (fb) in tissues such as the ventral prosencephalon (vp) and pharyngeal endoderm (pe). (B) At stage 15, the prosencephalon has divided into the telencephalon (te) and the diencephalon (di); at this stage *Shh* transcripts are localized to the diencephalic neuroectoderm (ne). (C) At stage 17, *Shh* is now expressed in the telencephalon. (D) Around stage 20, *Shh* is expressed in a new domain, the facial ectoderm (fe) in addition to being expressed in the diencephalic and telencephalic neuroctoderm. (E) *Shh* expression remains constant in the neuroectoderm and facial ectoderm from stage 22. (Panels reproduced courtesy of the *Journal of Clinical Investigation*.)

or undergrowth of the midface), cleft lip and palate, and a single incisor (Belloni et al. 1996; Schimmenti et al. 2003). Individuals at the mild end of the spectrum can have normal or near-normal appearing faces.

For a number of years it was assumed that the severity of the craniofacial malformations directly correlated with the severity of the brain malformation (Demyer et al. 1964). Neuroimaging studies and the ability to detect a number of mutations in HPE genes have shown that this relationship does not always exist, and has also led to a genetic classification of HPE. For example, patients with the most severe, or alobar, HPE brain malformations do not always exhibit cyclopia but may have facial malformations of intermediate severity. Conversely, patients with a mutation in an HPE gene such as *SHH* may reveal normal brain imaging results yet still exhibit facial malformations of intermediate severity. In addition, patients from the same family with identical mutations in *SHH* can exhibit differences both in brain and in facial manifestations, and yet other individuals with identical mutations may fail to meet the traditional or anatomical criteria for HPE.

Temporal perturbations in *Shh* signalling in avian models may help to explain the range of phenotypes associated with HPE

Given this absence of a clear genotype–phenotype correlation, it remains unclear how genetic alterations in *SHH* can lead to the various craniofacial phenotypes seen in HPE. It has been suggested that the production of an HPE phenotype requires multiple hits: in other words, not only an underlying genetic mutation, but also subsequent environmental influences such as teratogens must act simultaneously to produce HPE-related malformations (Ming & Muenke, 2002). Based on this idea, we hypothesized that the spectrum of HPE phenotypes may be related to the developmental stage at which subsequent teratogenic insults occur (Cordero et al. 2004). We chose the chick as a model system and Shh as our signalling molecule of interest to explore this possibility.

We used the steroidal alkaloid cyclopamine to block Shh signal transduction by exposing chick embryos to this agent at select timepoints governed by the dynamic *Shh* induction pattern (Cordero et al. 2004). Cyclopamine is a steroidal alkaloid extracted from the plant *Veratrum californicum* (Keeler, 1970, 1978; Incar-

dona et al. 1998) that has the ability to alter the protein conformation of a downstream component of the Shh signalling pathway, Smo, by binding to it (Chen et al. 2002a,b). In doing so, cyclopamine inhibits a series of intracellular processes that normally culminate in Shh signalling (Chen et al. 2002a,b).

Variable facial phenotypes recapitulating those observed in the human HPE spectrum were observed following administration of cyclopamine at different developmental timepoints (Cordero et al. 2004). Inhibition of Shh signalling in gastrulation-stage chick embryos produced forebrain malformations and cyclopia with a proboscis, as observed by other investigators (Incardona et al. 1998) and as seen in *Shh*-null mice (Chiang et al. 1996). When stage-15 embryos were treated with cyclopamine, prior to initiation of *Shh* expression in the telencephalon, as well as abnormal forebrain development craniofacial malformations consisting of microcephaly, microopthalmia, hypotelorism and hypoplasia of the maxillary primordia were noted (Cordero et al. 2004). Cyclopamine administration at stage 17, after the induction of *Shh* in the telencephalon but prior to its expression in the facial ectoderm, failed to produce gross brain anomalies (Cordero et al. 2004). In addition, the facial malformations resulting from treatment at stage 17 were less severe than those resulting from treatment at earlier stages (Cordero et al. 2004). The facial dysmophology consisted of mild hypotelorism and truncation of the distal upper beak (Cordero et al. 2004) (Fig. 7C), which is analogous to cleft lip and palate in humans. Evaluation of the skeletal elements revealed that truncation of the distal upper beak was secondary to a small premaxilla, which was aberrantly positioned ventral to the nasal capsule (Cordero et al. 2004) (Fig. 7D).

Molecular analyses revealed that the facial malformations were not secondary to neural crest apoptosis, but instead were due to molecular mispatterning in the facial ectoderm (Cordero et al. 2004). As mentioned, blocking Shh signalling at stage 15 led to the failure of *Shh* induction in the telencephalon and facial ectoderm, whereas treatment at stage 17 led to the failure of induction of *Shh* in the facial ectoderm domain (Cordero et al. 2004). The loss of the *Shh* expression domain in the facial ectoderm after treatment at these stages was accompanied by an ectopic proximal expression of the *Fgf8* domain from the FEZ (Cordero et al. 2004). As a result of this shift, the organizing properties of the FEZ (Hu et al. 2003) are lost.
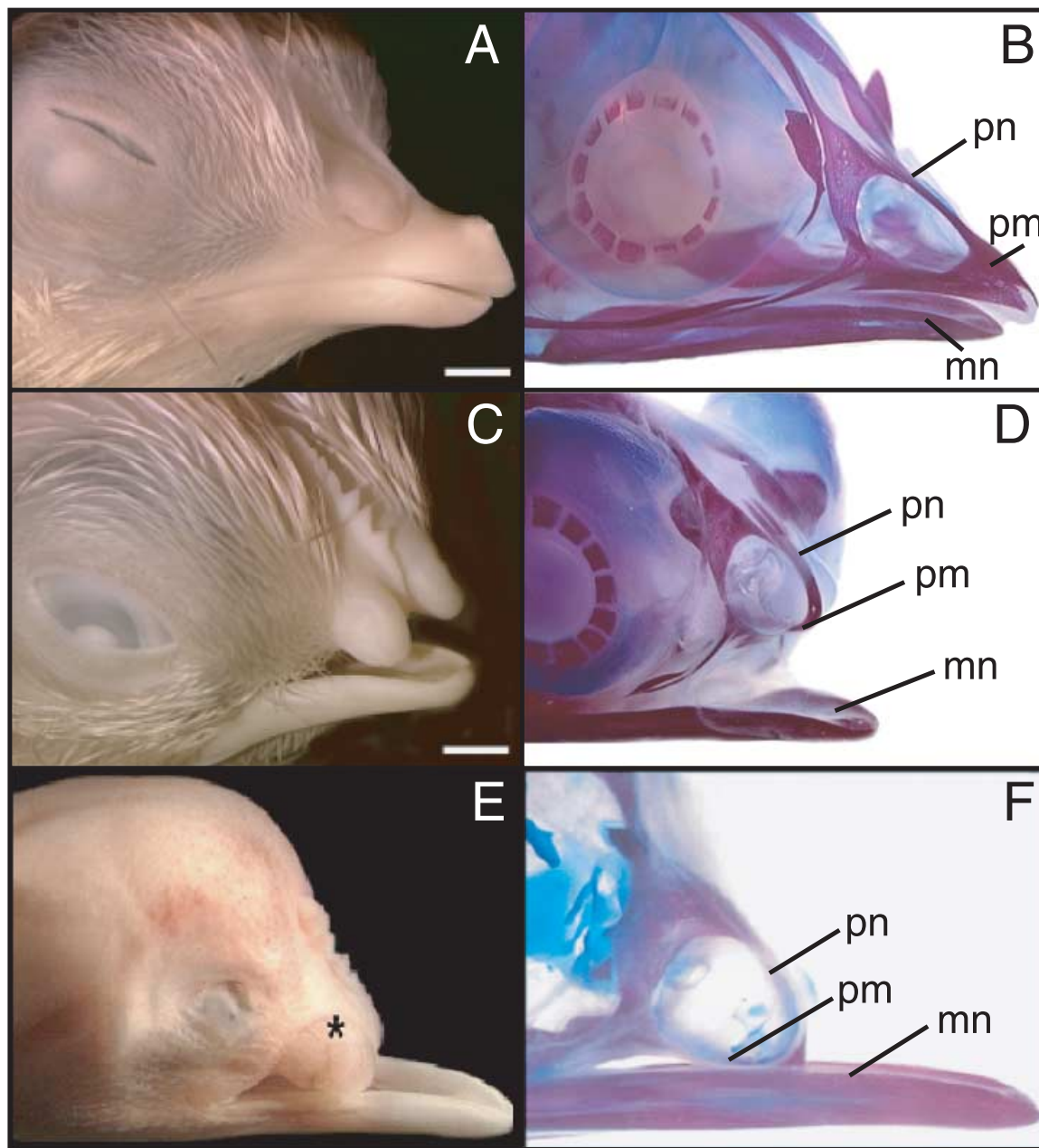
Fig. 7 Consequences of inhibition of Shh signalling. (A,C,E) Oblique views of stage-41 chick embryos; (B,D,F) alcian blue- and alizarin red-stained skeletal structures of these embryos. (A,B) Control embryos exhibit normal morphology and skeletal development. (C,D) When stage-17 embryos are exposed to cyclopamine, they exhibit mild microcephaly and hypotelorism, along with malpositioning and truncation of the distal upper beak [e.g. the body of the premaxillary bone (pm) is shifted ventrally and shortened]. Proximal elements such as the dorsal component of the premaxillary bone remain unaffected [e.g. the nasal process of the premaxilla (pn) is intact]. The mandible (mn) is unaltered in cyclopamine-treated embryos. (E,F) Injection of 5E1 cells, which produce antibodies to Shh at stage 9.5, results in microcephaly, abnormal eye development and abnormalities of the distal upper beak. The gross phenotype resulting from 5E1 injection (which blocks Shh emanating from the neuroectoderm) is reminiscent of the morphological changes resulting from cyclopamine treatment; compare with C and D. (Panels A–D reproduced courtesy of the *Journal of Clinical Investigation*; panels E and F reproduced courtesy of *Developmental Biology*.)

One drawback of cyclopamine is that it inhibits Shh signal transduction in a number of tissues. We therefore sought to determine the effects on craniofacial morphology by specifically blocking Shh emanating from the forebrain. Hybridoma cells producing antibody to Shh (5E1) were injected into the brains of stage-9.5 chick embryos (Marcucio et al. 2005). Blocking Shh from the forebrain via this approach resulted in

facial phenotypes seen following cyclopamine administration at stage 17 (Fig. 7E), and like cyclopamine was not secondary to neural crest cell apoptosis but from a molecular alteration in the FEZ leading to truncation of the upper beak, ventralization of the premaxillary bone and decreased expansion of the medial–lateral axes (Marcucio et al. 2005) (Fig. 7F). The resultant less severe phenotype in the 5E1 embryos treated at stage 9.5 as compared with the more severe phenotypes seen with cyclopamine at earlier stages reflects the ability of cyclopamine to diffuse into multiple tissues and pan-inhibit Shh in those tissues.

These data suggest that Shh plays an important role in 'linking' the brain and face during certain earlier developmental time points, and Shh signalling thereby provides evidence that the brain–face relationship is more than just a structural relationship based on physical proximity between these two important structures. However, at later stages after *Shh* has been established in the telencephalon the brain and face appear to develop independently. This temporal dependence and subsequent apparent independence between the brain and face during embryonic development may account for the incongruous presentations of HPE seen in clinical practice (e.g. some patients present with both brain and facial malformations, whereas other patients exhibit normal brain morphology despite facial malformations). However, it remains unclear whether *SHH* exhibits this dynamic pattern of expression during human development.

Shh from the epithelia is required for neural crest survival

Clearly, a definite correlation exists between proper timing of *Shh* expression in epithelial tissues and normal craniofacial development. But what cellular processes does Shh affect at these crucial developmental timepoints in order to ensure proper craniofacial development? As already discussed, Shh signalling clearly impacts cell proliferation and outgrowth of the frontonasal process. But moulding the craniofacial primordia into facial structures requires not only cell proliferation but also the induction of cell death in appropriate places, and perhaps more importantly, the survival of cells in other key places. Two seminal studies indicate that Shh signalling may be of importance in this regard as well.

Sara Ahlgren and colleagues noticed that the phenotype (e.g. Fetal Alcohol Syndrome) resulting from maternal alcohol consumption during pregnancy is incredibly similar to the phenotype that results from blocking Shh signalling (Ahlgren et al. 2002). Embryos in which Shh is blocked or which have been exposed to ethanol both display reduction of the frontonasal process, hypoplastic branchial arches and apoptosis of neural crest cells (Ahlgren et al. 2002). The similarity between phenotypes suggested to them that Shh may be mediating cell survival during craniofacial development. To that end, they examined the amount of *Shh* expression after ethanol exposure, and found that expression of a number of genes in the Shh signalling pathway, including the Shh receptor *Ptc* and its downstream effector *Gli*, are reduced after ethanol treatment (Ahlgren et al. 2002). Retroviral expression of *Shh* resulted in a dramatic decrease in the amount of cell death, along with proper formation of the frontonasal process, thereby rescuing the phenotype and confirming the role of Shh as a cell survival factor (Ahlgren et al. 2002). This finding is in accord with more studies that demonstrate that Shh is also required for the survival of neuroepithelial cells early in development (Thibert et al. 2003).

Ahlgren's conclusions that Shh is an important mediator of cell survival were reinforced by studies from Andrew McMahon's laboratory. McMahon removed Shh responsiveness from neural crest cells specifically by generating *Wnt-1-Cre*; conditionally null *Smoothened* (*Smo*) mice (*Wnt-1-Cre; Smo n/c*) (Jeong et al. 2004). Embryos carrying the conditional knockout of Smo, a downstream component of the Shh pathway, exhibit extensive loss of many neural-crest-derived craniofacial structures, as well as a down-regulation of Hedgehog targets [such as certain Forkhead (Fox) transcription factors] in the neural crest of these structures (Jeong et al. 2004). Perhaps more significantly, removal of Shh signalling from neural crest cells resulted in increased apoptosis and decreased cell proliferation in the arches, which manifests as a reduction of the frontonasal process in embryos that survive (Jeong et al. 2004). These results echoed Ahlgren's conclusion that Shh is necessary for cell survival, and also accords with new studies that demonstrate the role of Shh in skull development, as discussed later.

Shh is also required for chondrogenesis in the skull and in the lower face

Sculpting the craniofacial complex is a complex process. It requires the neural crest cells that form skeletal

elements not only to survive and proliferate, but also then to differentiate appropriately. As recent studies reveal, the omnipresent Shh is implicated even in the later differentiation of neural crest cells.

Thomas Schilling made these discoveries via elegant experiments in zebrafish (Wada et al. 2005). By creating a *sox10:egfp* transgenic zebrafish, he and colleagues were able to follow neural crest cells as they migrated and differentiated into craniofacial structures such as the anterior neurocranium, which consists of a medial ethimoid plate flanked by trabeculae on each side (Wada et al. 2005). Then, by mating this *sox10:egfp* fish with the *sonic you* (*syu*) fish in which *shh* signalling is disrupted, Schilling was able to determine what happens to these neural crest cells when Shh signalling is disrupted (Wada et al. 2005).

In normal embryos, the neural crest cells destined to become anterior neurocranium lie widely separated from one another across the ventral midline (Wada et al. 2005). These crest cells form condensations at later stages, and then differentiate into the cartilages (trabeculae and ethimoid) that comprise the anterior neurocranium (Wada et al. 2005). The transgenic *sox10-egfp;syu* mutant exhibited significant defects in formation of the anterior neurocranium, including a failure of the presumptive trabecular neural crest cells to separate (Wada et al. 2005). This lack of separation was traced to fusion (as opposed to apoptosis) of neural crest cells across the ventral midline, which led to a midline condensation that formed a single rod of cartilage (e.g. a fused trabeculae) (Wada et al. 2005).

Schilling suspected that Hedgehog signalling might have different actions on neural crest cells depending on the stage at which it exerts its effects (Wada et al. 2005). To inhibit Shh signalling at discrete timepoints, he and colleagues turned to cyclopamine (Wada et al. 2005). Similar to Cordero et al., Schilling found that the severity of the phenotype was dependent on the stage at which Shh was interrupted (Wada et al. 2005). Disruption of Shh with cyclopamine at earlier stages caused a complete loss of the anterior neurocranium, whereas disruption of Shh at later stages eliminated the medial ethmoid plate, while sparing the trabecular cartilages (Wada et al. 2005). Thus, these studies offered unequivocal confirmation that the range of HPE-associated phenotypes may reflect stage-dependent requirements for Hedgehog signalling (Wada et al. 2005).

What cellular processes does Shh signalling affect in order to produce these phenotypes? The answer was elucidated by applying exogenous Shh via mRNA misexpression at early stages in wild-type embryos, and via bead implantation at later stages in both wild-type embyos and mutants (Wada et al. 2005). Application of exogenous Shh after migration promoted chondrogenesis in the anterior neurocranium in normal embryos (Wada et al. 2005). Application of exogenous Shh in *sox9-egfp;syu* mutants rescued the phenotype and restored separation of the presumptive trabecular neural crest (Wada et al. 2005). Taken together, these data supported a role for Hedgehog signalling at later stages to promote chondrogenesis in the elements of the anterior neurocranium.

Other recent papers have also found that Shh plays an integral role in promoting chondrogenesis from mesenchymal condensations in other areas of the face. For instance, differentiation of condensations into Meckel's cartilage in the mandible has been shown to be Hedgehog dependent (Melnick et al. 2005). Noting the absence of mandibular structures in *Shh*-null mutants, Tina Jaskoll and colleagues undertook *in vitro* culture studies of stage E11–12 mandibular explants (Melnick et al. 2005). They found that application of cyclopamine to these cultures exhibit a stage-dependent inhibition of Meckel's cartilage chondroblast differentiation to mature chondrocytes (Melnick et al. 2005). Other studies by Cliff Tabin, however, have applied exogenous Shh alone to culture preparations and *in vivo* without finding evidence of increased chondrogenesis in the frontonasal prominence (Abzhanov & Tabin, 2004). Rather, they find that while Shh alone may not promote chondrogenesis in the midface, the FEZ domain of *Shh* juxtaposed with *Fgf8* serves to promote chondrogenesis and chondrogenic outgrowth during cranial development (Abzhanov & Tabin, 2004). These results suggest that Shh must act synergistically with other molecules, such as Fgf8, to produce chondrocyte differentiation and proliferation (Abzhanov & Tabin, 2004).

Future directions

Although Shh has gained prominence in recent years as one of the major mediators of craniofacial morphogenesis, this molecule is by no means the only player involved. As mentioned, Shh acts in conjunction with Fgf8 to initiate outgrowth and chondrogenesis of the frontonasal process. Other studies investigating retinoic acid signalling in the face also highlight Fgf8 as a molecule of interest.

More recently, several papers have highlighted the role of bone morphogenetic proteins (Bmps) in producing outgrowth of the frontonasal prominence, and have suggested that differences in Bmp levels may be responsible for species-specific differences the appearance of the frontonasal prominence (Abzhanov et al. 2004; Wu et al. 2004). Yet other data are beginning to hint that Wnts may also mediate craniofacial development (Niemann et al. 2004; S. Brugmann, unpublished observations). The role of these molecular pathways in craniofacial development has yet to be elucidated in detail; additionally, how these molecules interact with Shh and Fgf8 has yet to be determined. Given the complexity and individuality inherent in faces, both within and among species, it is certain that craniofacial development requires a complex interplay between all of these molecular pathways.

Now that it is known that molecular signalling in the face might involve a number of molecular pathways, an equally intriguing question emerges: namely, how are all these molecular signals coordinated between tissues? For example, hypotelorism can be elicited by disrupting signals emanating from the neuroectoderm, but it can also be caused by disrupting signals from the facial ectoderm (Cordero et al. 2004). These findings suggest that both the brain and the face are sources of patterning signals. But how are these signals from the brain and face coordinated? How much crosstalk actually occurs between the brain and the face, and at what developmental timepoints does it occur? Is the neural crest involved in mediating crosstalk between the neuroectoderm and facial ectoderm, or does it merely passively respond to signals from these tissues? If the crest actually participates in the signalling, what molecules in the neural crest relay signals between these tissues? Finding the answers to these questions will be crucial to gaining a complete picture of craniofacial development, and in learning how to repair congenital craniofacial defects.

Perhaps an equally pressing line of inquiry concerns *Hox* genes. While understanding molecular signalling is crucial to preventing birth defects, further studies on *Hox* genes may help facilitate regenerative processes of craniofacial healing. As mentioned, *Hox* genes imbue neural crest cells with a positional code that directs them to take residence in particular arches. Evidence exists that depending on the particular *Hox* code present, the neural crest cells adopt different degrees of fates, and possess different degrees of plasticity that enable them to form different facial structures. It remains to be determined whether these *Hox* codes are retained into adulthood, and if so, whether that information can be used to give cells the plasticity necessary for craniofacial repair and regeneration after injury. All of these questions will provide intriguing areas of exploration.

Conclusion

Although it has been resolved that the neural crest plays an enormous role in the production of facial structures, some neural crest populations require instructions from their local environment. In instances where a Hox code dictates plasticity, the complex signals come from the local milieu of the superficial ectoderm, the neuroepithelium and the pharyngeal endoderm. Much of the crosstalk that occurs between these tissues during early embryonic development involves common signalling pathways such as the Shh, Fgf, Wnt and Bmp pathways. These molecular dialogues appear to impact upon several cellular processes, including neural crest survival, proliferation and differentiation. An equally important aspect of this crosstalk is that it occurs at very specific developmental timepoints, and outside these timepoints there appear to be tissue-specific developmental mechanisms that are independent of other tissues. The dependence and independence shared by the brain and face at different timepoints may explain why brain and facial malformations are often seen together, but also how abnormal facial development can occur in the presence of an apparently normal brain.

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